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Original Article

ANTIVIRAL ACTIVITY OF *SAUSSUREA LAPPA* ETHANOL EXTRACT AGAINST SARS-COV-2: *IN VITRO* STUDY

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ABSTRACT

Objective: The study aims to investigate the antiviral activity of *S. lappa* against Severe Acute Respiratory Syndrome-Coronavirus 2 (SARS-CoV-2) *in vitro*.

Methods: The extracts are obtained by ultrasonic-assisted extraction (UAE) with a 96% ethanol solvent. Thin-layer chromatography (TLC) uses n-hexane: ethyl acetate and chloroform: methanol) as mobile phases. The staining outcome is subsequently examined using UV visualizers with a wavelength of 366 nm. To assess the antiviral activity of Vero E6 cells, extracts were employed at doses of 25, 50, 75, and 100 µg/ml, with remdesivir serving as the positive control. Supernatants were collected on days 1, 2, 3, and 6 for qRT-PCR testing with target genes E and ORF1ab. Time-addition experiments were conducted to determine how the extract works as antiviral. Protein expression was tested with Western blots with antibodies S and N SARS-CoV-2.

Results: TLC identifies terpenoid chemicals present in the ethanol extract of *S. lappa.* The ethanol extract of *S. lappa* exhibited antiviral effects against SARS-CoV-2, with an inhibitory concentration 50 (IC_{50}) of 40 µg/ml, a cytotoxic concentration 50 (CC_{50}) of 131.4 µg/ml, and a selectivity index of 3.51. The extract can potentially impact the entry-post-entry phase of SARS-CoV-2 infection in Vero cells. The immunoblotting results demonstrated a reduction in the expression of S and N proteins in the treatment group compared to the negative control.

Conclusion: *S. lappa* ethanol extract has antiviral activity against SARS-CoV-2 based on an *in vitro* study.

Keywords: *Saussurea lappa*, SARS-CoV-2, Antiviral activity

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INTRODUCTION

In the last decade, there has been an increase in the prevalence of viral infections worldwide, one of which is the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection [1]. The SARS-CoV-2 pandemic was officially announced by the World Health Organization (WHO) on March 11, 2020 [2]. Global research endeavors have been undertaken to discover remedies for the epidemic, resulting in novel breakthroughs in fundamental and applied research, treatment, diagnostics, vaccine development, and epidemiology [3].

Clinical research has focused on several approved antiviral drugs, including remdesivir [4], favipiravir [5], and molnupiravir [6]. Several medicinal plants have been shown to have an inhibitory effect on viral infections *in vitro* and *in vivo*, thus making them candidate for aid in the treatment of SARS-CoV-2 [7, 8]. Plants have a variety of secondary metabolites such as flavonoids, terpenoids, lignans, tannins, and alkaloids that have a range of anti-infectious activities and antioxidants [9]. Several secondary metabolites are used as antivirals, including phenolates, carotenoids, terpenoids, and alkaloids [10].

Saussurea lappa is a plant that is being considered as a potential antiviral candidate. According to literature studies, the analysis of *S. lappa* shows the presence of several metabolites, including sesquiterpenes, flavonoids, phytosterols, lignans, and terpenes. Some of the most important chemicals that have been isolated are costunolide lappadilactone, dihydrocostunolide, and dehydrocostus lactone [11, 12]. Myrcene is a chemical in *S. lappa* that can interact with the angiotensin-converting enzyme 2 (ACE2) receptor. This receptor is responsible for allowing the entry of SARS-CoV-2 into host cells. The anti-inflammatory capabilities of *S. lappa* can be utilized in treating COVID-19 by mitigating the levels of inflammatory cytokines such as TNF-α and IL-1β [13]. Exploration of a plant metabolite's potential in revolutionizing COVID-19 treatment offers a promising avenue for therapeutic development. Previous research focused on investigating the effects of *S. lappa* on SARS-CoV-2, but it was still unable to explain the mechanisms of the extract as an antiviral. This study investigates the novel impact of this natural compound on SARS-CoV-2, which has not been previously explored.

MATERIALS AND METHODS

S. lappa root extraction

Saussurea lappa root was obtained as a sample in the pharmaceutical biology laboratory at the Department of Pharmacy, Faculty of Medicine and Health Sciences, UIN Maulana Malik Ibrahim, Malang, with reference number 075/212/10220-A/2022. Simplisia *S. lappa* is extracted using the UAE (Ultrasonic-Assisted Extraction) method with a 96% ethanol solvent. The extraction results are filtered using filtered paper and dialed with nitrogen gas, thus obtaining a concentrated extract. The extract is collected, applied in a rotary evaporator to obtain a thick extract, and then dried in the oven at a temperature of 40 °C [14].

Cells and virus

Vero E6 cells cultivated in Dulbecco's modified Eagle's medium (DMEM, GIBCO-INVITROGEN) containing 100 g/ml penicillin, 100 g/ml streptomycin (Sigma) and 5% Fetal Bovine Serum (FBS, GI BCO-Invitrogen), at 37 °C in a CO2 incubator of 5%, Bovine serum albumin (BSA, Roche), TritonX-100 (Promega), Formaldehyde

(HCHO, Applicam), Tripsin EDTA (GIBCO-Invitrogen). A 100% confluent Vero cell will be used in this experiment. The SARS-CoV-2 isolate was obtained from the SARS-CoV-2 collection at the Institute of Tropical Diseases, Airlangga University, Surabaya, Indonesia. The variant used is B.1.470, which was the most common variant in Indonesia as of June 1, 2021 [15].

Thin layer chromatography (TLC)

The extract is dissolved in 1 ml of 96% ethanol and then infused on a silica gel plate F254 and then diluted using the motion phase of n-hexane eluent: ethyl acetate and chloroform: methanol. The emerging spots were observed using ultraviolet (UV) visualizers at wavelengths of 254 nm and 366 nm. Next, silica gel plates are sprayed with H_2SO_4 10% v/v, and heated to 105 °C for 5 min using a TLC plate heater. The stain outcome was subsequently examined using an UV visualizer with a wavelength of 366 nm [16].

Cytotoxicity test

The extract's cytotoxicity towards cells was assessed using the 3-(4.5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetra-sodium bromide test (MTT). Vero E6 cells were cultured in 96-well plates and treated with samples of varying concentrations. The cells were then incubated for 48 h. Following incubation, the medium was extracted and subsequently supplemented with 15 µl of MTT reagent. The mixture was then incubated for an additional 4 h. Subsequently, 100 µl of dimethyl sulfoxide (DMSO) was used to dissolve the residues that had developed due to the MTT reaction. The GloMax Microplate Multidetection Reader (Promega) was used to measure absorption at 560 nm and 750 nm wavelengths. Vero cell viability percentage values are analyzed, and cytotoxicity concentration 50 (CC_{50}) values are obtained with probit analysis [17].

Antivirus activity test

Testing was done by seeding the Vero cells on the 6-well plate. Extracts with 25; 50; 75; and 100 µg/ml concentration are prepared in ependorf, with Remdesivir 10 µM as a positive control, respectively. Then, each extract concentration was added to the virus isolate, as much as 200 TCID50. Remove the medium from the 6-well plate, then add a mixture of extract and virus, shaking the plate so that the virus and extract are even. Then put 6-well plates in a CO_2 incubator at of 37 °C. Observations were performed on days 1, 2, 3, and 6 with a reverse microscope to see the percentage of cells experiencing cytopathic effects. The supernatant from each treatment is collected on days 1, 2, 3, and 6 and subsequently subjected to quantitative testing. Extracting RNA by Reverse Transcription Polymerase Chain Reaction (qRT-PCR) involves using the QIAmp Viral RNA Mini Kit (manufactured by Qiagen, Germany). The detection of viral genomic material is then carried out using the One Step TaqMan real-time RT-PCR kit (manufactured by Thunderbird, Toyobo, Japan) [18].

Time addition experiment

To determine the mechanism of the extract's inhibition against SARS-CoV-2, a time addition experiment was conducted with three mechanisms: (1) Entry-post entry mechanism is achieved by adding *S. lappa* extract along with viral inoculation for 1 hour and then for a 24-hour period of infection, (2) Entry step inhibition, in which the virus and test material are innoculated on the cell for 2 h, then incubated for 24 h with the added medium, (3) Post-entry inhibition is the way the virus is inoculated into the cell, then incubated for 24 h with the addition of *S. lappa* extract [19]. Supernatants of each treatment are harvested on days 1; 2; 3; and 6, followed by qRT-PCR and Western blot analysis [19, 20].

Immunoblotting

The infected Vero cells are injected with a RIPA buffer, then run with SDS-polyacrylamide gel electrophoresis (SDS page) and transferred to a polyvinilidene membrane (PVDF) (Milipore, Bedford, MA, USA). The membrane was then incubated with a primary monoclonal anti-spike mouse (GTX632604; GeneTex, 1:5000) and an anti-nucleocapsid monoclonal mouse. (GTX632269, GeneTex, 1:2000) [20].

Statistical analysis

Analysis of data from the cytotoxic test with the MTT method obtained absorbance data from each concentration of the test substance. Cytotoxicity concentration of 50% (CC_{50}) is determined by probit analysis using SPSS software. Antiviral test data is obtained from the Ct value, which is analyzed with Probit using SPSS software to obtain the IC_{50} value. Analysis of the barrier mechanism by calculating the Ct values of each entry stage, post-entry, and entry-post-entry at a specific concentration of substance compared to the control data were obtained in the form of means and standard deviations. Statistically significant differences between groups are determined using one-way analysis of variance (ANOVA) with a p-value of less than 0.05. The expression of S and N proteins is calculated using ImageJ software.

RESULTS

Thin layer chromatography (TLC) analysis of extracts of *Saussurea lappa*

The results of the characterization of chemical compounds with TLC show that 96% of the ethanol extract from the *S. lappa* root contains terpenoid compounds, specifically triterpenoids, that are shown with a change in stain color to purple after heating, as shown in fig. 1.

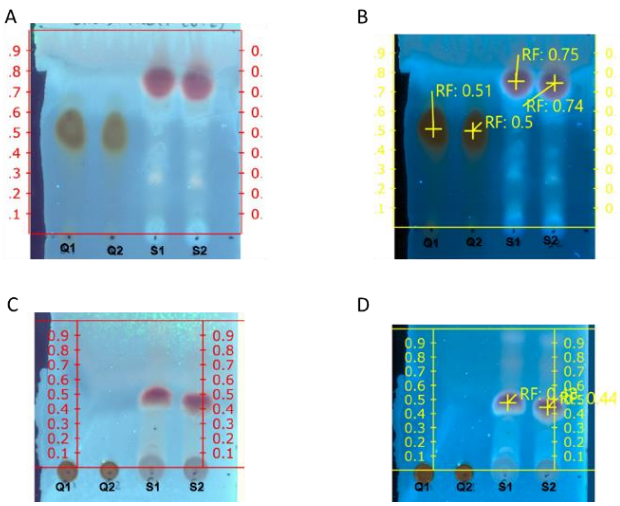

Fig. 1: Thin layer chromatography (TLC) test results on 96% ethanol extract of *S. lappa*. GF254 silica gel stationary phase, the mobile phase (A) CHCl3:MeOH; (B) N-hexana: etyl acetate, and quercetine as control. (A) and (C) observed under UV 254 nm; (B) and (D) observed under UV 366 nm. Q1-Q2 = quercetin, S1-S2 = *S. lappa* ethanol extract

Cytotoxic test

Toxicity testing of the test material was performed on Vero E6 cells without SARS-CoV-2 administration, and then absorption measurements were performed at wavelengths of 560 nm and 750 nm. In this test, a cytotoxicity concentration of 50 (CC_{50}) of 131.4 µg/ml was obtained.

Anti-SARS-CoV-2 activity ethanol extract (96% *S. lappa*)

Antiviral activity tests of 96% *S. lappa* ethanol extract were conducted using Vero E6 cells and SARS-CoV-2 virus variant B.1.470. Supernatants from each treatment were collected on days 1, 2, 3, and 6, then a polymerase chain reaction test with target genes E and ORF1ab was conducted to determine the Ct value. This test resulted in a 50% inhibition concentration of 40 µg/ml. The selectivity index (SI) describes the ratio of the cytotoxicity (CC_{50}) to the antiviral activity (IC_{50}) of a drug. A higher SI indicates how effective a drug is for a specific virus infection [21]. This means that the higher the SI, the greater the chance that the test material will developed into a product. The SI value of ethanol extract 96% root *S. lappa* is obtained at 3.51.

Based on fig. 2, the results of the Ct value can be seen on each day of observation in different test groups. Different test results show that the variable Ct values between the test groups have significant differences ($p<0.05$).

Table 1: Results of the Ct value of *S. lappa* ethanol extract activity against SARS-CoV-2 with target gene E

Groups	Ct values (Average)±SD*			
	Day 1	Day 2	Day 3	Day 6
100 µg/ml	28.90±0.035	26.38±0.021	26.15±0.078	26.54±0.042
75 µg/ml	26.03±0.219	18.95±0.989	17.30±0.002	16.93±0.028
50 µg/ml	26.70±0.085	17.00±0.020	16.93±0.028	14.80±0.021
25 µg/ml	25.15±0.169	16.73±0.035	13.48±0.078	10.80±0.007
Negative control	27.57±0.332	16.21±0.014	12.19±0.002	7.29±0.113
Remdesivir	28.18±0.057	20.56±0.071	18.86±0.063	17.95±0.092

*SD value (Standard Deviation) 3 times replication

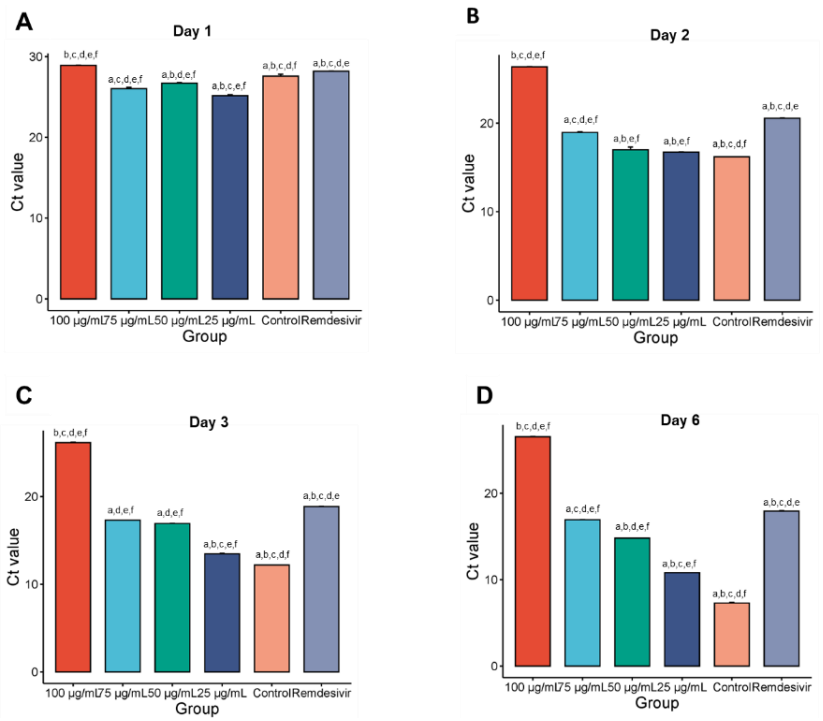

Fig. 2: Ct value of *S. lappa* ethanol extract against SARS-CoV-2 on days 1, 2, 3, and 6 of observations with target gene E

Table 2: Results of the Ct value of *S. lappa* ethanol extract activity against SARS-CoV-2 with target gene ORF1ab

Groups	Ct values (Average)±SD*			
	Day 1	Day 2	Day 3	Day 6
100 µg/ml	28.29±0.08	25.94±0.01	25.41±0.17	26.54±0.04
75 µg/ml	25.47±0.07	18.95±0.10	18.95±0.09	16.93±0.03
50 µg/ml	26.24±0.08	18.95±0.09	15.71±0.17	14.80±0.02
25 µg/ml	24.85±0.19	16.73±0.04	12.62±0.00	10.80±0.01
Negative control	26.95±0.12	15.86±0.05	11.16±0.13	7.29±0.11
Remdesivir	27.59±0.20	20.57±0.05	19.13±0.05	17.95±0.09

*SD value (Standard Deviation) 3 times replication

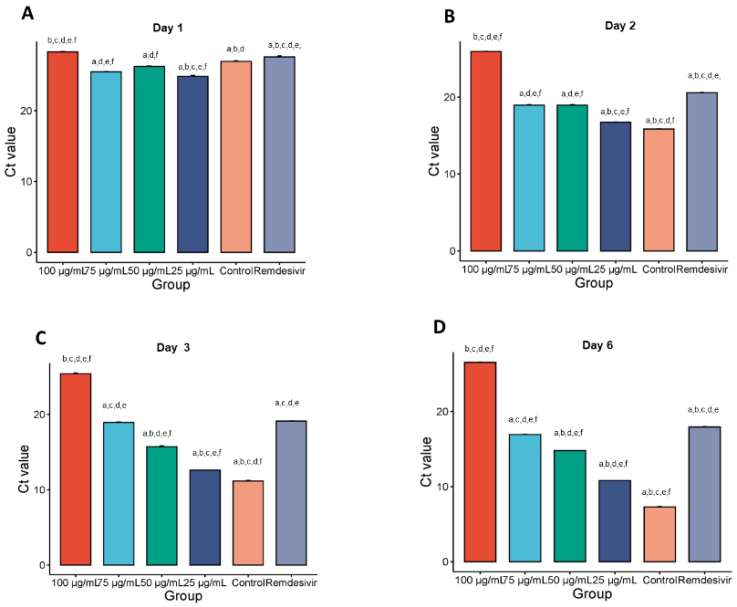

Fig. 3: Ct value of *S. lappa* ethanol extract against SARS-CoV-2 on days 1, 2, 3, and 6 of observations with target gene ORF1ab

The results demonstrated that *S. lappa* ethanol extract has activity as an antiviral for SARS-CoV-2 by assessing the Ct value with target Gen E and ORF1ab (fig. 2 and 3). Compared to virus control, *S. lappa* ethanol extracts can increase Ct values significantly depending on the dose and day of observation (p-value<0.05). However, at a dose of 100 µg/ml, the extract produces toxic effects on Vero E6 cells, so from the RT-PCR results, a higher Ct-value than the positive control is obtained (Remdesivir).

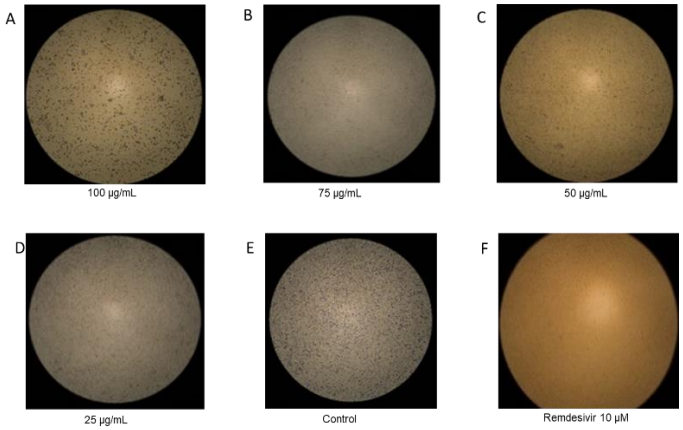

Fig. 4: Observation results on day 3, with the microscope inverted to see cells experiencing cytopathic effects. (A) shows the presence of toxic effects of extracts on E6 Vero cells; (B), (C), (D), and (E) show cytopathic effects characterized by cell rounding and detachment of cells; (F) shows no occurrence of cytopathic effects

Time addition experiment

The results of the time addition experiment, as shown in fig. 5, show a difference between the result of the Ct value on each day of observation and the stage of the working mechanism of the test material. On the observation of the first day, there are significant differences between the results of Ct values in each treatment group (p<0.05), where the entry-post entry mechanism obtains the highest value. The same results were found on days 2, 3, and 6.

Table 3: Ct value of time addition experiment based on day observation with target Gene E

	Ct values (Average)±SD*			
Day	**Entry-post entry (++)**	**Entry (+-)**	**Post entry (-+)**	**Negative control**
1	30.62±0.205	28.96±0.042	26.03±0.219	27.77±0.002
2	26.70±0.085	24.85±0.198	20.56±0.070	17.39±0.028
3	20.57±0.070	19.16±0.049	18.25±0.002	11.53±0.177
6	16.68±0.023	13.00±0.318	11.53±0.177	6.97±0.127

*SD value (Standard Deviation) 3 times replication

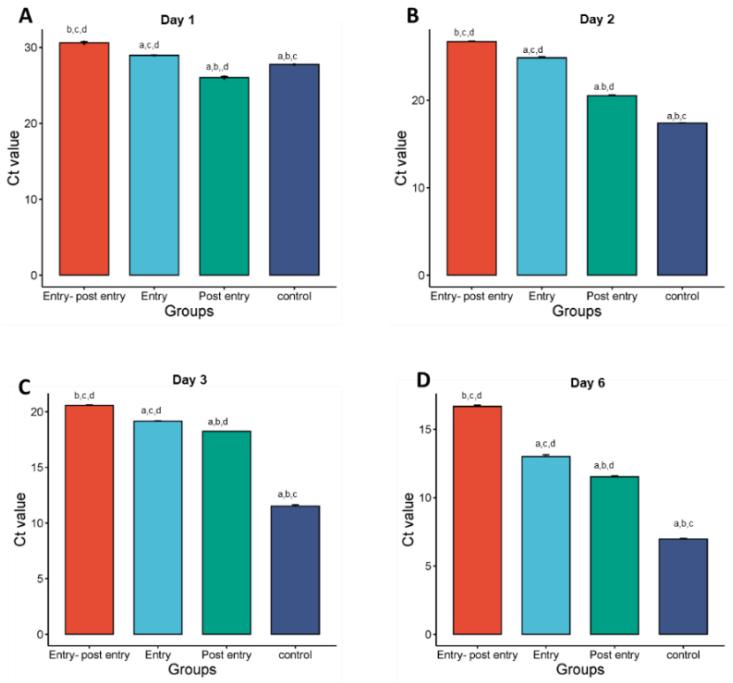

Fig. 5: Time addition experiment with target gene E

Table 4: Ct value of time addition experiment based on day observation with target Gene ORF1ab

	Ct values (Average)±SD*			
Day	**Entry-post entry (++)**	**Entry (+-)**	**Post Entry (-+)**	**Negative control**
1	30.36±0.45	28.33±0.05	25.56±0.01	27.41±0.04
2	26.24±0.08	24.85±0.19	20.57±0.07	16.91±0.20
3	20.56±0.07	19.16±0.05	18.25±0.04	10.61±0.09
6	16.68±0.23	12.93±0.08	10.61±0.09	6.03±0.16

*SD value (Standard Deviation) 3 times replication

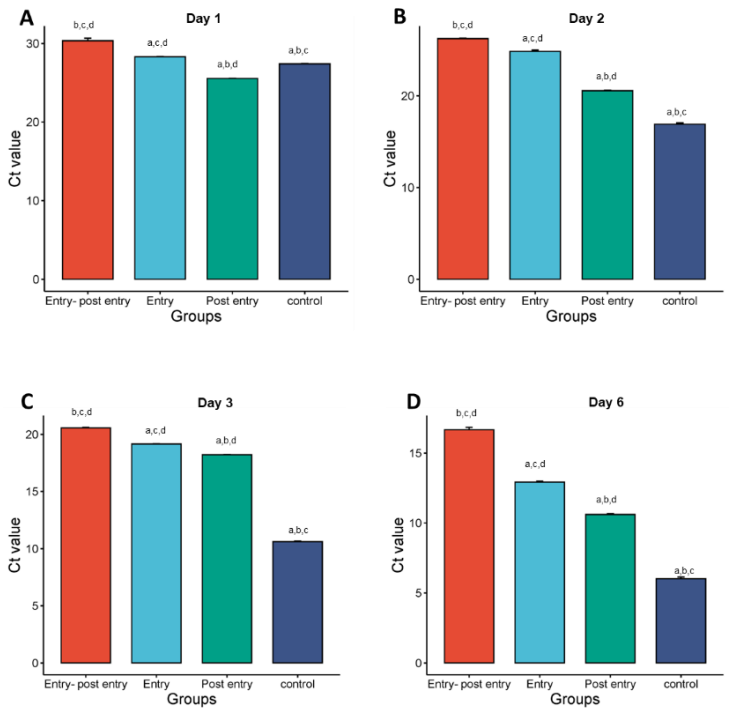

Fig. 6: Time addition experiment with target gene ORF1ab

Time addition experiments with target genes based on correlation tests (fig. 7) show strong negative correlations between Ct value results and observation days, both on entry-post entry and entry and post-entry mechanisms. This suggests that the longer the observation day, the lower the Ct values, both in the treatment and control groups.

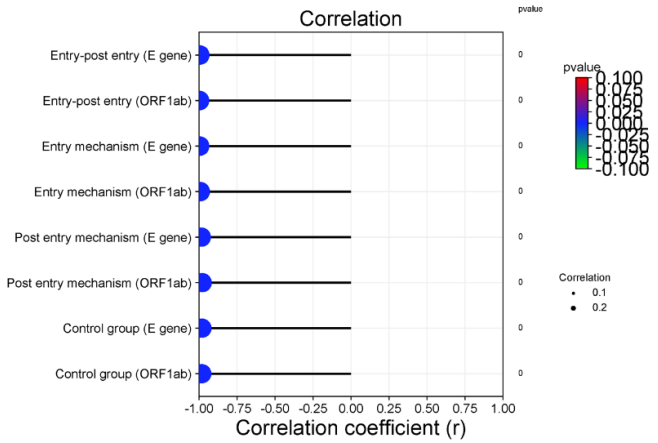

Fig. 7: Correlation of Ct value result with observation day

Based on the correlation test (fig. 8), a strong positive correlation exists between the dose of *S. lappa* extract and the Ct value on day two observation, with a degree of significance of 0.001. Similarly, the results of Ct values on days 3 and 6 of observation show a strongly positive correlation between the extract dose and the result of the Ct value, with a degree of significance of 0.000. This indicates that the higher the dose, the higher the Ct value.

However, on day one observations, it showed a weak positive correlation, with a degree of significance of 0.272 (target gene E) and 0.267 (target gene ORF1ab).

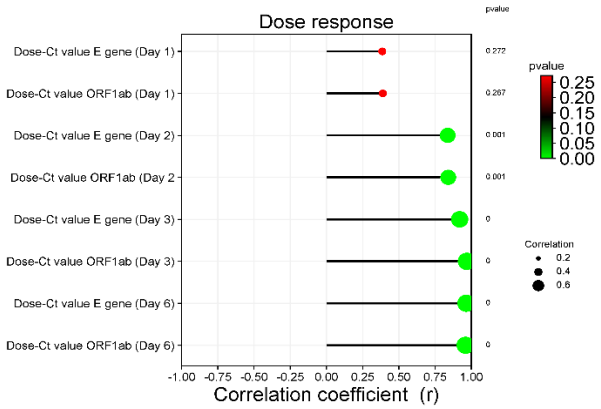

Fig. 8: The dose correlation of the extract with the resulting Ct value on observation days 1, 2, 3, and 6

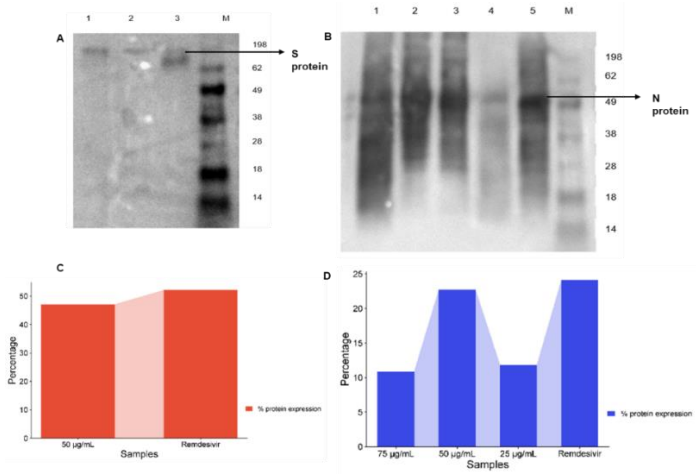

Fig. 9: Protein expression in S and N SARS-CoV-2. (A) The result of a western blot with the antibody Protein S; (1) 50 µg/ml; (2) remdesivir; (3) negative control; (B) The result of a western blot with the antibody Protein N; (1) 75 µg/ml; (2) 50 µg/ml; (3) 25 µg/ml; (4) Remdesivir; (5) negative control; (C) Protein S expression; (D) Protein N expression

Immunoblotting

In the time addition experiment, it is known that *S. lappa* ethanol extract is likely to work at the entry-post entry stage, so it is necessary to test the expression of proteins produced by viruses, especially proteins related to the identification stage, such as protein S, and proteins associated with virus replication, namely protein N. The result of the protein expressions S and N is shown in fig. 9.

Quantitative analysis of protein expression using ImageJ software showed a decrease in S protein expression by 52.1% in the positive control group (Remdesivir) and by 47.1% in extract concentrations of 50 µg/ml compared to the control group. Decreases in N protein expression were also shown in the remdesivir group as well as the extract groups of 25, 50, and 75 µg/ml, respectively, by 24.1%, 11.8%, 22.7%, and 10.9%.

DISCUSSION

The bioactivity of medicinal plants is affected by the chemical content. In this study, the TLC results showed that *S. lappa* ethanol extract includes terpenoid components. This aligns with the literature study of *S. lappa*, which contains various variable metabolites such as sesquiterpenes, flavonoids, phytosterols, lignans, and terpenes. Sesquiterpenes are the most abundant compounds in *S. lappa*, with costunolide and dehydrocostus lactone being the main active ingredients [22, 23]. Multiple investigations have demonstrated that terpenoids tend to function as inhibitors of SARS-CoV-2 [24]. The myrcene compound present in *S. lappa* impacts the ACE-2 receptor, hence impeding the entry of SARS-CoV-2 into the host cells [25].

The antiviral activity was analyzed to see how well the ethanol extract of *S. lappa* protected Vero E6 cells from infection with SARS-CoV-2. The results showed that the extract can inhibit SARS-CoV-2, with IC_{50} values of 40 µg/ml. Other studies have indicated that *S. lappa* extract has partial antiviral activity with effectiveness of 75%, 65%, and 13% against the SARS CoV-2/Wuhan strain B.1.36 at concentrations of 1/64, 1/128, and 1/256 [26]. While the toxicity test also showed that *S. lappa* ethanol extract had a CC_{50} of 131.4 µg/ml, this result was similar to previous reports that showed *S. lappa* ethanol extracts had a CC_{50} of 162.9 µg/ml [27] and 70.36±0.8 µg/ml [28]. The selectivity index (SI) for this study was 3.51 points. An ideal substance as an antiviral is one whose cytotoxic concentration is high and the inhibitory concentration of the antivirals is low [29].

This study also explains a positive correlation between the extract dose and the Ct value, where the higher the extraction dose, the higher the Ct value when compared to the control group. This study aligns with the research conducted by Ulbegi Polat *et al.* (2023), which discovered a positive correlation between the concentration of extracts and the percentage of antiviral activity. In other words, as the concentration of the extracts increased, the level of antiviral activity also increased [26]. An experiment was conducted to discover the specific stage of the virus cycle in which the extract exhibits antiviral action. The extract is introduced at various stages throughout the infection to achieve this objective. Fig. 5 and 6 demonstrate that the ethanol extract of *S. lappa* exhibits both virucidal action, preventing virus entry, and inhibitory effects on the virus once it has entered host cells. This is demonstrated by obtaining a higher Ct value at the entry-post entry level compared to the entry and post-entry levels alone. Other research suggests that a water extract from *Saussurea costus* can inhibit the virus once it enters the host cells [30]. There was a strong negative correlation between the results of the Ct value and the day of observation, suggesting that the longer the observation day, the lower the Ct value, which means that the extract activity against the virus will decrease.

The Western blot analysis of immunoblotting revealed a reduction in the expression of S and N proteins in the treatment group compared to the negative controls. Protein S is linked to virus recognition, whereas protein N is a structural protein (SARS-C0V-2) that facilitates the packing of genetic material, assists in the folding of RNA, transports proteins inside cells, breaks down DNA, and restricts the host's immunological response [31, 32]. Protein N, the most abundant protein in virus-infected cells, is characterized by its genetic stability, making it a promising candidate for therapeutic targeting [33].

CONCLUSION

Thin-layer chromatography results obtained terpenoid compounds from *S. lappa* ethanol extract. The ethanol extracts of *S. lappa* demonstrate antiviral properties against SARS-CoV-2, with a cytotoxic concentration (CC_{50}) of 131.4 µg/ml and an inhibitory concentration (IC_{50}) of 40 µg/ml. This results in a selectivity index of 3.51. It is possible that *S. lappa* extracts work at the entry-post entry stage against SARS-CoV-2, as well as a decrease in the expression of proteins S and N in the treatment group compared to the negative control.

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AUTHORS CONTRIBUTIONS

AAF: concept and design, literature search, experimental studies, data acquisition, data analysis, manuscript preparation, manuscript editing; DS: concept and design, data acquisition, data analysis, manuscript preparation; DJAJALAKSANA: concept and design, data acquisition, data analysis, manuscript preparation; SSK: concept and design, data acquisition, data analysis, manuscript preparation; RM: concept and design, experimental studies, data acquisition, data analysis, manuscript preparation, manuscript editing; MIL: concept and design, experimental studies, data acquisition, data analysis, manuscript preparation; SRP: concept and design, data acquisition, data analysis, manuscript preparation

CONFLICT OF INTERESTS

There is no conflict of interest among all the authors.

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